Journal of Clinical Virology 57 (2013) 59–65

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Genetic characterization of human respiratory syncytial virus detected in hospitalized children in the Philippines from 2008 to 2012

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ARTICLE INFO

Article history:
Received 25 September 2012
Received in revised form
13 December 2012
Accepted 2 January 2013

Keywords:
Respiratory syncytial virus
Childhood pneumonia
Genotype
The Philippines

ABSTRACT

Background: Human respiratory syncytial virus (HRSV) is the leading cause of acute lower respiratory tract infection in infants and young children. However, molecular characteristic of HRSV is still unknown in the Philippines.
Objective: To describe the molecular epidemiology of circulating HRSV detected in the Philippines.
Study design: From May 2008 to April 2012, nasopharyngeal swabs were collected from infants and children aged between 7 days and 14 years who were hospitalized with severe pneumonia. HRSV was detected by nested PCR targeting M2 gene, and C-terminus of the G gene was sequenced for phylogenetic analysis.
Result: Out of total 2150 samples, 19.3% ($n = 415$) were positive for HRSV, and 65.0% of them ($n = 270$) were identified as HRSV-A and 35.0% ($n = 145$) as HRSV-B. There were two major HRSV outbreaks: between June 2008 and February 2009, and between June and March 2012. Majority of HRSV strains detected during the former outbreak were HRSV-A (97.5%, 203/208) whereas during the later outbreak, both HRSV-A (54/158, 34.2%) and HRSV-B (104/158, 65.8%) were detected. All HRSV-A strains were classified as genotype NA1 and all HRSV-B as genotype BA, which had 60-nucleotide duplication in secondary hypervariable region of the G gene. Among HRSV-B positive samples, there were 2 distinct clusters with unique amino acid changes and low homology in compared to other strains in BA, suggesting emergence of new variant of HRSV-B.
Conclusion: The study provides an overview of the genetic variation in circulating HRSV viruses in the Philippines along with identification of possibly a novel variant of HRSV-B.

1. Background

Acute lower respiratory tract infection (ALRI) is a leading cause of death in children aged less than five years worldwide, and most of those deaths occur in developing countries.[1] The role of human respiratory syncytial virus (HRSV) in the etiology of ALRI has been well defined in developed countries[2,3] as well as in developing countries.[4–7] In the Philippines, several etiological studies on ALRI in children have been conducted, in which HRSV were detected in 7.1–18.9% of the ALRI cases.[8–12] Recently, we showed that HRSV is the second most common respiratory viral pathogen next to rhinoviruses among the children with severe pneumonia in the Philippines.[13] However, molecular characterization of the detected HRSV has not been yet studied.

HRSV has been divided into two subgroups (HRSV-A and HRSV-B) by reactivity with monoclonal antibody.[14,15] The HRSV envelope contains three transmembrane surface glycoproteins; the major attachment protein G, the fusion protein F, and the small hydrophobic SH protein. G protein is mainly involved in virus attachment

Abbreviations: HRSV, human respiratory syncytial virus; HRSV-A, human respiratory syncytial virus subgroup A; HRSV-B, human respiratory syncytial virus subgroup B; ALRI, acute lower respiratory tract infection; EVRMC, Eastern Visayas Regional Medical Center; NPS, nasopharyngeal swab; IMCI, Integrated Management of Childhood Illness; VTM, viral transport medium; RITM, Research Institute for Tropical Medicine; cDNA, complementary DNA; N, nucleocapsid; m-PCR, multiplex polymerase chain reaction; M-MLV reverse transcriptase, Moloney murine leukemia virus reverse transcriptase.

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1386-6532
http://dx.doi.org/10.1016/j.jcv.2013.01.001

to host cells and plays major role in the immunogenicity of the virus. Nowadays, HRSV subgroups can also be identified by genetic analysis.[16] G gene is the most variable among HRSV genes and has two hypervariable regions. The analysis of this region is commonly used for studying the molecular epidemiology studies of HRSV.[17,18] Currently, HRSV-A has 10 genotypes (GA1–GA7, SAA1, NA1, NA2) whereas HRSV-B has 9 genotypes (SAB1–SAB4, GB1–GB4, BA) with BA having 11 branches (BA1–11).[19–25]

2. Objectives

The objective of this prospective study was to describe the molecular epidemiology of circulating HRSV in the Philippines for the last four years between May 2008 and April 2012 by phylogenetic analysis of the secondary hypervariable region of G gene.

3. Study design

3.1. Patients and clinical samples

From May 2008 to April 2012, nasopharyngeal swabs (NPSs) were collected from infants and children aged between 7 days and 14 years who were hospitalized for severe pneumonia in Eastern Visayas Regional Medical Center (EVRMC) in Tacloban city of Leyte island, the Philippines.[13] The patients were diagnosed as severe pneumonia according to the case definition of Integrated Management of Childhood Illness (IMCI).[26] A total of 2150 patients were enrolled into the study. After sample collection, NPSs were stirred in 3 ml of viral transport medium (VTM) and transported with ice packs to the Research Institute for Tropical Medicine (RITM) in Manila for further analysis.

3.2. RNA extraction and complementary DNA synthesis

RNA was extracted from supernatant of clinical samples using QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany). Reverse transcription PCR to synthesize complementary DNA (cDNA) was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase and random primers (all were from Invitrogen Carlsbad, CA, USA).

3.3. Detection of HRSV

For HRSV screening, multiplex polymerase chain reaction (m-PCR) was performed to amplify nucleocapsid (N) gene of HRSV together with influenza A virus, influenza B virus, and human metapneumovirus as previously described (Table 1).[13,27] The results of multiplex PCR were confirmed by PCR targeting M2 gene using Ex Taq (TaKaRa, Otsu, Japan) (Table 1).[28] From January 2010, we used PCR targeting M2 gene as an alternative screening method.

3.4. Subgrouping, sequencing and phylogenetic analysis of HRSV

To determine subgroups, we performed the nested PCR targeting C-terminus of the G gene, which included the secondary hypervariable region (HRSV-A: 270 bp, HRSV-B: 330 bp)[17,22] (Table 1). After purifying the amplification product using SUPREC™ PCR (TaKaRa, Otsu, Japan), nucleotide sequence was determined by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA, USA).

Related sequences were searched by using the Basic Local Alignment Search Tool (BLAST). Phylogenetic trees based on sequences of the second hypervariable region of G gene were generated by the neighbor-joining method and maximum composite likelihood model with Molecular Evolutionary Genetic Analysis (MEGA) software version 5.0.[29] Since there is an insertion of 60 nucleotides in HRSV-BA, we used both complete and pairwise deletion method for treating the gap and confirming the consistency of formulated trees. Representative sequences of the corresponding region of each genotype were adapted from previous studies,[20–25,30,36–39] and the identical sequences were excluded from the analysis (Supplemental Table 1). *Homogeneity* among the strains was compared by pairwise distances (p-distance) estimated by MEGA software version 5.0. All sequences used in present study were deposited in the GenBank under accession numbers: AB749604–AB749760.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2013.01.001.

4. Results

4.1. Detection and subgrouping of HRSV

From May 2008 to April 2012, 2150 NPSs were collected from hospitalized children with severe pneumonia and 19.3% ($n = 415$) were positive for HRSV. HRSV positive rates were varied yearly with the highest rate in 2008 (33.0%, 203/615) and the lowest in 2009 (1.8%, 6/339) (Fig. 1). By subgroup specific nested PCR targeting G gene, HRSV-positive samples were classified into HRSV-A (65.0%, 270/415) and HRSV-B (35.0%, 145/415).

During the study period, there were two major HRSV outbreaks; one was in between June 2008 and February in 2009 and another one was in between June 2011 and March 2012. Majority of HRSV strains detected in the earlier outbreak were HRSV-A (203/208, 97.5%). On the other hand, the outbreak in 2011 to 2012 was caused by both HRSV-A (62/164, 37.8%) and HRSV-B (102/164, 62.1%) (Fig. 1). Notably, in the beginning of the second outbreak in 2011, a majority of detected HRSV was HRSV-A, but the numbers of HRSV-B cases were increased toward the end of the outbreak. From October 2009 to March 2010, only HRSV-A cases were detected; on the other hand, both HRSV-A and HRSV-B were detected between May 2010 and January 2011. It is noteworthy that increased numbers of total hospitalized cases with severe pneumonia coincided with these two HRSV outbreaks. Only sporadic cases were detected in between October 2009 and January 2011 with slight increase in January 2010.

4.2. Genetic characterization

The sequence of the second hypervariable region of C-terminus of G gene was determined for 96 out of 270 (35.6%) HRSV-A positive samples and 77 out of 145 (53.1%) HRSV-B positive samples (Supplementary Table 2).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2013.01.001.

The phylogenetic tree of G gene's second hypervariable region (270 nucleotides) indicated that all HRSV-A were classified as NA1[24] (Fig. 2A). Based on the BLAST, the cases detected in 2008–2009 had high homology (>97.6%) with other NA1 strains detected in different parts of the world. NA1 strains detected in 2011–2012, except TTa-11-0405, and two strains in 2010 (TTa-10-0261 and TTa-10-0328) were appeared to form a unique cluster under NA1. Three amino acid substitutions, Asp^{237}, Leu^{274}, and Ser^{292} have been previously reported as specific amino acid substitutions for NA1.[24,25] We also found these substitutions in most of the NA1 strains analyzed in the present study such as Asp^{237} in 94.2% (82/96), Leu^{274} in 94.2% (82/96), and Ser^{292} in 100% (96/96) (Fig. 4A). Another specific substitution for NA1, an early stop codon at position 298, was also observed in 100% (96/96) of NA1 strains. In addition to these previously reported NA1 specific substitutions,

Table 1
Primers used for detection and analysis for human respiratory syncytial viruses (HRSV) at Eastern Visayas Regional Medical Center (EVRMC) from 2008 to 2012.

	Primer	Sequence (5′ → 3′)	Target gene	Reference
Multiplex PCR	vrs P1	GGAACAAGTTGTTGAGGTTTATGAATATGC	Nucleocapsid gene	26
	vrs P2	TTCTGCTGTCAAGTCTAGTACACTGTAGT		
	vrs i	GGTGTACCTCTGTACTCTC		
Detection of HRSV	22K1	ATGTCACGAAGGAATCCTTGC	M2 gene	27
	22K2	TAGCTCTTCATTGTCCCTCAGC		
	22K3	GAGGTCATTGCTTAAATGG		
	22K4	GCAACACATGCTGATTGT		
Genotyping of HRSV	GPA	GAAGTGTTCAACTTTGTACC	G gene	19
	GPB	AAGATGATTACCATTTTGAAGT		
	nRSAG	TATGCAGCAACAATCCAA		21
	nRSBG	GTGGCAACAATCAACTCTGC		
	F1	CAACTCCATTGTTATTTGCC		

two additional amino acid substitutions, Ile^{253} and Tyr^{273} were also found in a unique cluster for the strains of 2011 and 2012.

The phylogenetic tree based on the same region for HRSV-B (330 nucleotides) indicated that all 77 HRSV-B strains were classified as BA, whose genotype could be further divided into 11 branches (Fig. 2B)[21,30], however the length of the sequences deposited in Genbank is too short that those BA11 strains were excluded from the analysis. There were only 3 analyzed strains of HRSV-B in 2008, which shared same node with BA10, but they were not classified into any of known BA1 to 11 deposited in Genbank (Fig. 2B). p-distances in between 2008 strains and BA branches were 0.038 ± 0.009, which suggesting that this is a new variant.[31] These strains had common sequence for BA7 to BA10 (Pro^{223}), and showed high homology with both BA7 and BA10 (98% and 97%, respectively) but not with specific sequence for BA10 (Gly^{292})[21] (Fig. 4B). Furthermore, they had common amino acid substitution (Gly^{251}), which had not been observed in BA7–BA10. From the above findings, we considered that those 2008 strains variants were diverged from BA10, and we tentatively named these variants as Cluster-A.

On the other hand, most of the strains detected from 2009 to 2011 were classified as BA9, and they had high homology (>97%) with other BA9 strains. Additionally, we also found specific substitution in most of the strains as previously reported, Ala^{271} in BA9 (94.3%, 66/70) (Fig. 4B)[20]. Interestingly, there were a total of 14 strains detected from July to December 2011, which appeared to be diverged from BA9. There were no sequences in Genbank which showed high homology (over 97%). p-distances between them and BA branches were 0.072 ± 0.01, suggesting a new variant.[31] We tentatively name this variant as Cluster-B (Figs. 2B and 4B). Moreover, Cluster-B had the common node with sharing 3 common unique amino acid substitutions (Pro^{219}, Lys^{242}, Pro^{257}) (Fig. 4B), and strains of this branch had same amino acid substitution (Ala^{271}), which was specific for BA9. Both clusters did not include strains which were reported in recent studies.

5. Discussion

In this study, HRSV was detected from 19.3% children who were hospitalized with severe pneumonia. The results confirmed the etiological importance of HRSV in hospitalized children with ALRI in the Philippines,[4–6,12,13] but the positive rates of HRSV in the present study were varied yearly during the study period, ranging from 33.3% in 2008 to 1.8% in 2009. The detection rate may fluctuate[25] or constant[18,31] depending on the study setting. Since the global dissemination of the pandemic A(H1N1)2009 occurred during the study period, the virus interference by pandemic A(H1N1)2009 might have an effect on the incidence of respiratory viruses including HRSV as observed in China.[32] However, in this study population, pandemic A(H1N1)2009 did not cause widespread outbreak in 2009. At least none of our study subjects were positive for influenza A(H1N1)pdm in 2009 (data not shown). Therefore, it is less likely that epidemic of HRSV in 2009 was interfered by pandemic A(H1N1)2009.

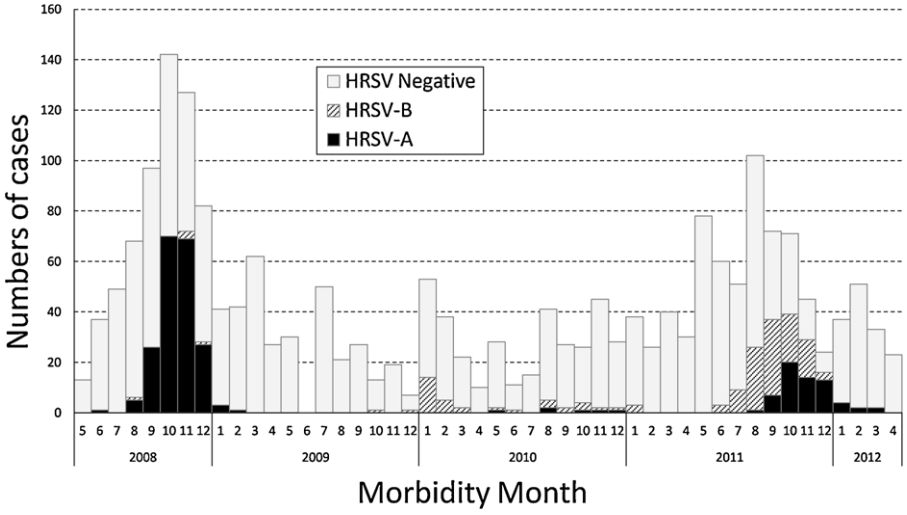

Fig. 1. Monthly distribution of numbers of hospitalized cases with pneumonia (white bar) and human respiratory syncytial virus (HRSV) positive cases (black bar)(A), and human respiratory syncytial virus subgroup A (HRSV-A, lattice pattern) and subgroup B (HRSV-B, slanted line) cases (B) at Eastern Visayas Regional Medical Center (EVRMC) from 2008 to 2012.

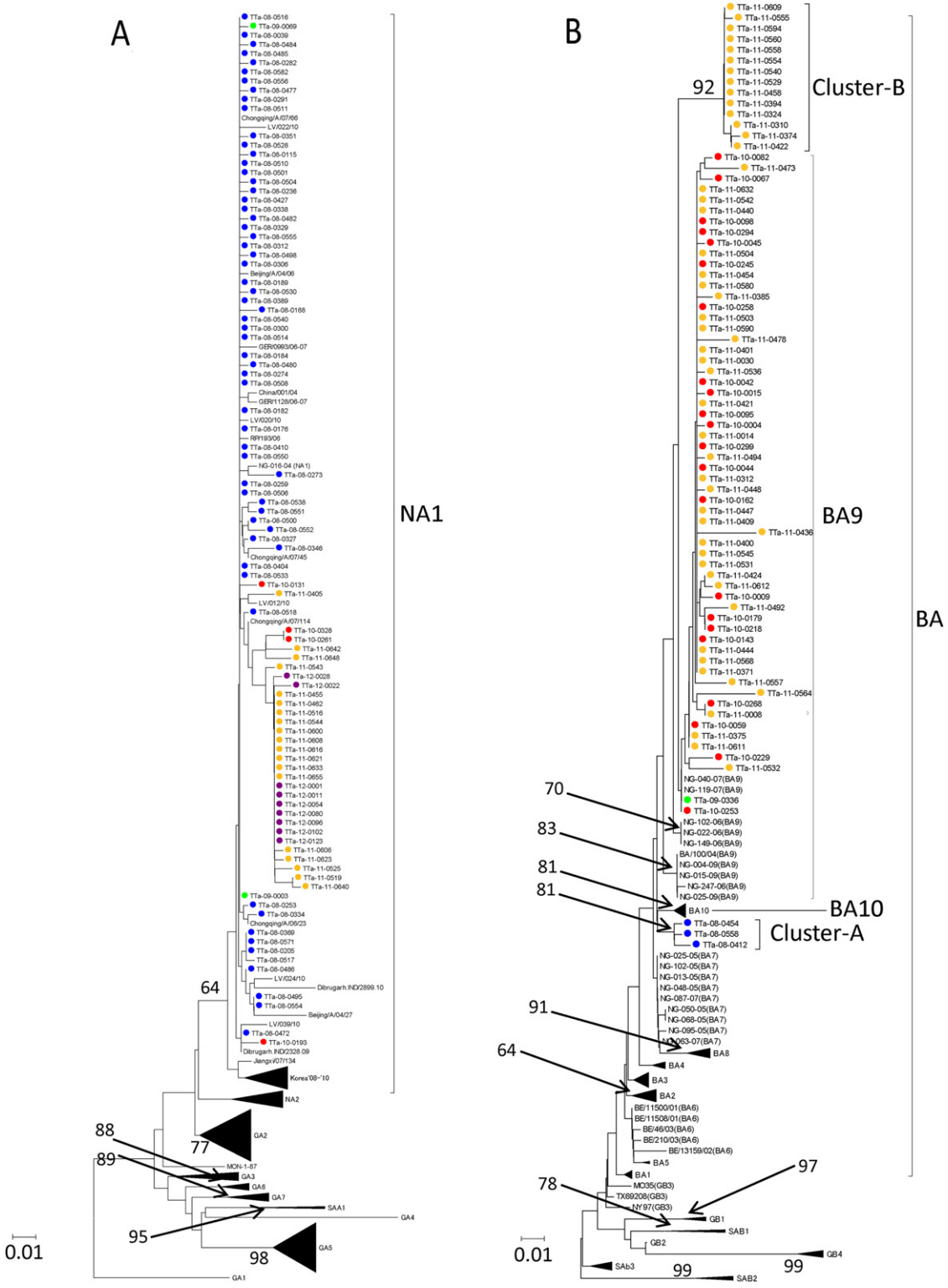

Fig. 2. Phylogenetic tree of human respiratory syncytial virus (HRSV) subgroup A (A) and subgroup B (B) detected in the Philippines from 2008 to 2012. Phylogenetic tree for subgroups A and B was constructed based on nucleotide sequence of the secondary hypervariable region of G gene using the neighbor-joining method with MEGA, version 5. Bootstrap value determined for 1000 interactions. Strains detected in 2008: blue, strains detected in 2009: green, strains detected in 2010: red, strains detected in 2011: orange, Strains detected in 2012: purple. BA: Argentina, BKK: Thailand, BR, Bra, RP: Brazil, Beijing, Chongqing, Jiangxi: China, mon: Uruguay, MO, CH, TX, WI: USA, MAD: Spain, PN, DEL, New Delhi. Dibrugarh.IND: India, Ger: Germany, JPN, NG: Japan, Ken: Kenya, LV: Latvia, US: USA, SAfrica: South Africa.

The genotyping of HRSV is important not only for classification of the viruses, but also for better understanding its molecular evolution and diversity. The second hypervariable region of G gene contains strain-specific epitopes and plays important roles in immune response. Furthermore, recent studies have reported that emergence of a variant may intensify the size of outbreak.[24] Therefore, monitoring of circulating genotypes of HRSV is imperative.

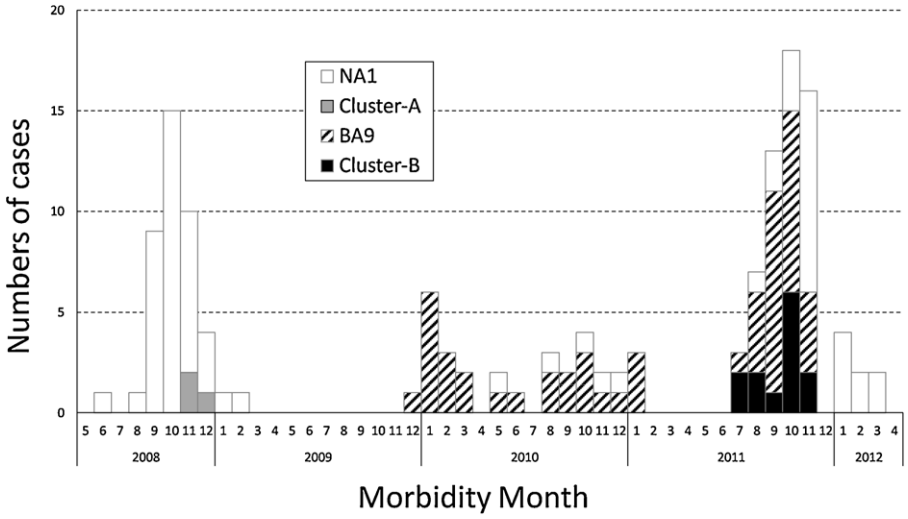

Fig. 3. Monthly distribution of human respiratory syncytial virus (HRSV) genotypes for HRSV-A (A) and HRSV-B (B).

Different patterns of predominance of HRSV-A and B were observed in different parts of the world. For example, the predominant group was changed in 2-year cycle in Finland,[33] but another study indicated that both HRSV-A and B were co-circulating in the same community.[34] NA1, the only genotype for HRSV-A found in the present study, was first identified in Niigata, Japan in 2004.[24] Recently published data from Cambodia, Korea and China indicated that a majority of HRSV-A viruses detected after 2006 were NA1.[25,35,36] These data together with our present findings suggest that NA1 has been predominantly circulating HRSV-A genotype at least in Asia between 2006 and 2012. Two strains in 2010 and all strains in 2012 (except one) formed a unique cluster under NA1,

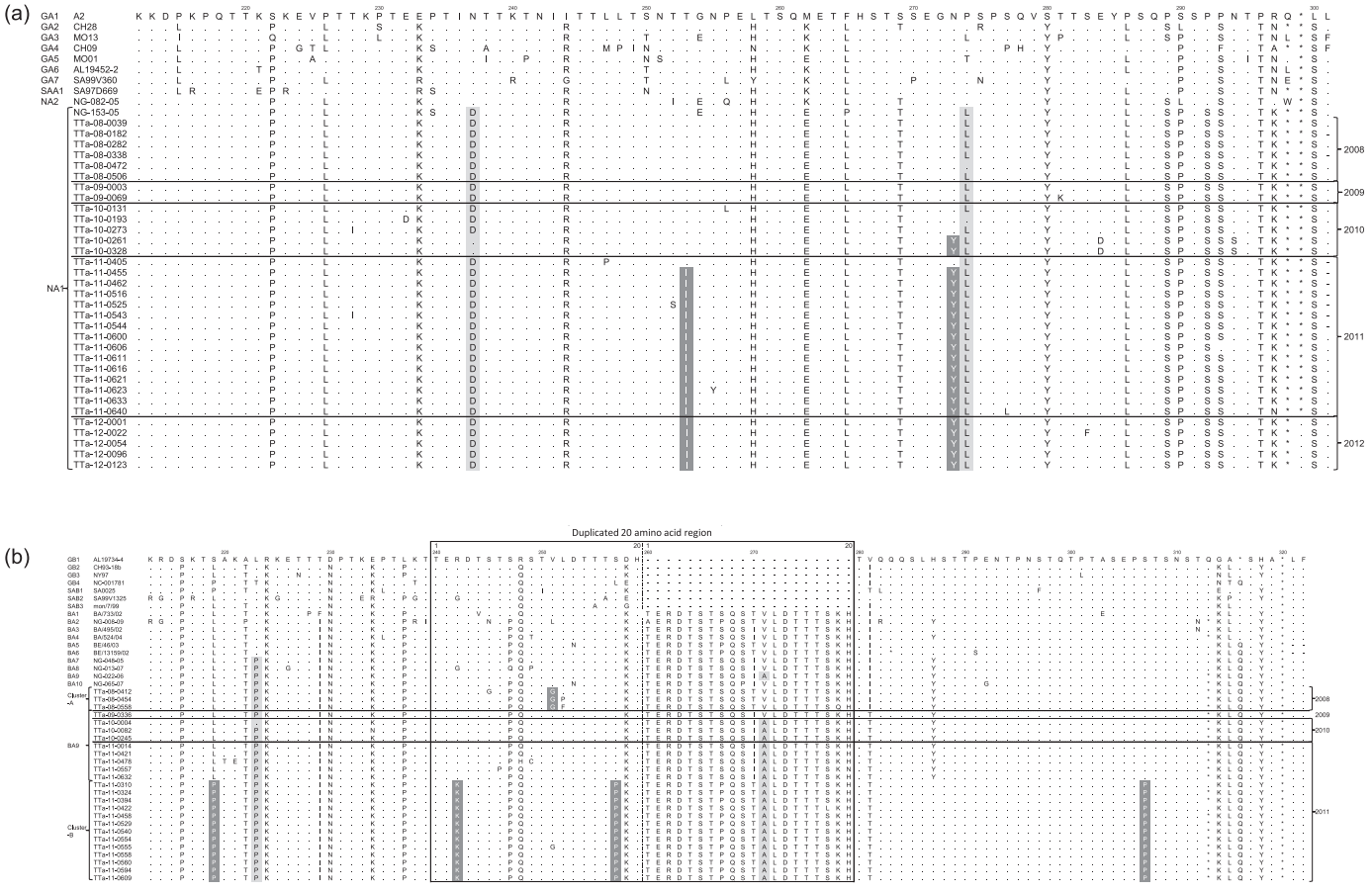

Fig. 4. Alignment of amino acid sequence for the secondary hypervariable region of human respiratory syncytial virus (HRSV) subgroup A (A) and HRSV subgroup B (B). Identical residues are indicated by dots, and stop codons are indicated by asterisks. No amino acids are indicated by hyphen inside of duplicated 20 amino acid regions. Light gray shows common amino acid in NA1 or BA7-BA10 or BA9, and gray shows common amino acid in 2011 strain of HRSV-A (90 amino acids) and Cluster-A and Cluster-B of HRSV-B (110 amino acids).

which was branched from strain in 2008 and 2009 and had two amino acid substitutions specific to this sub-clade. These findings suggest that further molecular evolution has been undergoing for NA1 in the Philippines.

All of HRSV-B strains analyzed were classified as BA, which was first identified in Buenos Aires, Argentina in 1999. It has a duplication of 60 nucleotides in the second hypervariable region of the G gene.[19] After its emergence in 1999, BA was spreading globally and became the predominant among the recently circulating HRSV-B strains in most of the countries.[30,37] During the global spread of BA, viruses had been evolving rapidly and created many new branches (at least 11 branches) under BA.[21,30,35] Among these BA branches, BA4 became a dominant branch after 2005.[30] However in our study, BA9 was a dominant BA branch from the end of 2009. BA9 was first identified during 2006-2007 season and became a predominant branch of BA during 2009–2010 season in Niigata, Japan.[21] BA9 was also the most common branch of BA in Korea between 2008 and 2010.[35] These findings indicate that BA9 has become a predominant BA branch at least in Asia by replacing other BA branches such as BA4 and BA5.

Owing to the viral evolution, there is a need to define recent global distribution of BA variants. We found potential variants under BA, which we tentatively named as Cluster-A and Cluster-B (Figs. 2B and 4B). Despite of our four years of observation period, Cluster-A strains were detected only in 2008. Given that they shared common sequences with BA7–BA10, having specific sequence, and sharing same node with BA10, they can possibly be a variant of BA10 branches.

In addition, we detected possible variant under BA9, which named as Cluster-B (Figs. 2B and 4B). Cluster-B was first identified in July 2011, and co-circulating with BA9 until the end of 2011. Cluster-B had specific amino acids substitution for BA9 and 3 unique amino acid substitutions. Therefore, we considered Cluster-B as a variant under BA9. Further follow up is required to observe the impact of emergence of these variants.

It was reported that multiple genotypes in each subgroup were co-circulating.[17,38] However, we observed quite simple genotype distributions in the present study. For HRSV-A, only single genotype, NA1 was found during the study period. BA9 was the single branch found in 2008 as well as from December 2009 and January 2011. Only a mixture of branches of BA, i.e. BA9 and Cluster-B was found in a cluster of cases in 2011. Our study site hospital, EVRMC is located in Leyte Island, which is a remote island in the Philippines. Such a geographic isolation may contribute to distribution of HRSV subgroups and genotypes. Further studies are therefore needed to define the subgroup and genotype distribution in rural settings in developing countries, where the impact of HRSV may be high.[13,39]

There are several limitations in our study. Firstly, we could not sequence all identified HRSV strains, which might be worthwhile to identify other genotypes, if any. Secondly, we conducted this study in one tertiary hospital in an island of the Philippines. Other places of the Philippines may have different distribution patterns of HRSV subgroup and/or genotype. Thirdly, even though we covered the last four years in our study, it may not be enough to draw any conclusion on the temporal distributions of subgroup and/or genotypes of HRSV in this region. Despite these limitations, this is the first molecular analysis on HRSV in the Philippines and our current study data contributes to understanding the global scenario of HRSV molecular epidemiology.

Funding

This work was supported by a grant-in-aid from the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan and Science and Technology Research Partnership for Sustainable Development (SATREPS) from Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA).

Ethical approval

The study protocol was approved by the Institutional Review Boards of RITM and EVRMC, and the Ethical Committee of Tohoku University Graduate School of Medicine. The children were enrolled in the study after taking written informed consent from their parents.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

We thank the staff of EVRMC and Tohoku-RITM Collaborating Research Center on Emerging and Reemerging Diseases.

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